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1 Agonism of free fatty acid receptors 1 and 4 generate peptide YY-mediated inhibitory responses in
2 mouse colon

3

4 **Short title:** FFA1 and FFA4 activities in mouse colon

5

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16

17 **Financial disclosures:** None.

18

19 **Statement of Conflicts of Interests:**

20 RM, IRT and HMC have nothing to disclose. DMS and MS are currently employed by and hold
21 shares in AstraZeneca Plc.

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27 Background and purpose:

28 Free fatty acid receptor 1 (FFA1) and FFA4 are located on enteroendocrine L cells with the
29 highest gastrointestinal (GI) expression in descending colon. Their activation causes the release of
30 glucagon-like peptide 1 and peptide YY (PYY) from L cells. Additionally, FFA1 agonism
31 releases insulin from pancreatic β -cells. As these receptors are modulators of nutrient-stimulated
32 glucose regulation, this study's aim was to compare the pharmacology of commercially available
33 agonists (TUG424, TUG891, GW9508) with proven selective agonists (JTT, TAK-875, AZ423,
34 Metabolex-36), in the mouse.

35 Experimental approach:

36 Mouse (>10 weeks old; C57BL/6J) mucosa was mounted in Ussing chambers, voltage-clamped
37 and the resultant short-circuit current (I_{sc}) recorded continuously. The pretreatments included
38 FFA1 antagonists or $Y_1 \pm Y_2$ antagonists. Glucose-sensitivity was investigated by mannitol
39 replacement apically and colonic and upper GI transit was assessed *in vitro* and *in vivo*.

40 Key Results:

41 FFA1 and FFA4 agonism required glucose and reduced I_{sc} in a PYY- Y_1 -dependent manner. The
42 novel compounds were more potent than GW9508. The FFA1 antagonists (GW1100 and
43 ANT825) blocked FFA1 activity only and revealed FFA1 tonic activity. The FFA4 agonist,
44 Metabolex-36 slowed colonic transit *in vitro* but increased intestinal transit *in vivo*.

45 Conclusion and Implications:

46 The selective FFA1 and FFA4 agonists were more potent than GW9508, a dual FFA1 and FFA4
47 agonist. We found that a PYY- Y_1 paracrine epithelial mechanism mediated their responses which
48 were glucose-sensitive, potentially limiting hypoglycaemia. ANT825 revealed tonic activity and
49 the possibility of endogenous FFA1 ligands causing PYY release. Finally, FFA4 agonism induced
50 regional differences in transit.

51 **Keywords:** Free fatty acid receptors 1 and 4, peptide YY, glucagon-like peptide 1, mucosal ion
52 transport, colonic transit

53

54

55 **Abbreviations**

- 56 ANOVA, analysis of variance;
- 57 ap, Apical;
- 58 AZ, AstraZeneca;
- 59 BIBO, BIBO3304;
- 60 BIIE, BIIE0246;
- 61 bl, Basolateral;
- 62 CCK, Cholecystokinin;
- 63 CNS, Central Nervous System;
- 64 DMSO, dimethyl sulphoxide;
- 65 FFA, free fatty acid;
- 66 FFA1, free fatty acid receptor 1;
- 67 FFA4, free fatty acid receptor 4;
- 68 GI, gastrointestinal;
- 69 GIP, Gastric inhibitory peptide;
- 70 GLP-1, glucagon-like peptide 1;
- 71 GLP-2, glucagon-like peptide 2;
- 72 GPCR, G protein-coupled receptor;
- 73 GSIS, Glucose stimulated insulin secretion;
- 74 HPMC, Hydroxylpropyl methylcellulose;
- 75 i.p., Intraperitoneal;
- 76 I_{sc} , Short-circuit-current;
- 77 KH, Krebs Henseleit;
- 78 KO, Knockout;
- 79 MC4 receptor, Melanocortin-4 receptor;
- 80 Met-36, Metabolex-36;
- 81 MMC, Myoelectric migrating complex;
- 82 OXM, oxyntomodulin;
- 83 Phlor, Phloridzin;
- 84 PLC, Phospholipase C;
- 85 PVP, polyvinylpyrrolidinone;
- 86 PYY, peptide YY;
- 87 PYY (3-36), peptide YY(3-36);

88 SDS, sodium dodecyl sulfate;
89 SGLT1, Sodium-glucose co-transporter 1;
90 SST, Somatostatin;
91 T2DM, type 2 diabetes mellitus;
92 UGIT, Upper gastrointestinal transit;
93 VIP; vasoactive intestinal polypeptide;
94 WT, wild type
95

96 **Introduction**

97 Free fatty acid receptors 1 ([FFA1](#), previously known as GPR40) and 4 ([FFA4](#), previously known
98 as GPR120) are modulators of nutrient-stimulated glucose regulation and therefore are therapeutic
99 targets for the treatment of type 2 diabetes mellitus (T2DM). FFA1 selective agonists have been
100 shown to cause glucose-sensitive insulin secretion (Tsujiata et al. 2011). FFA1 and FFA4 were
101 deorphanised in 2003 and 2005 respectively, as receptors for medium to long chain unsaturated
102 fatty acids (Hirasawa et al. 2005, Itoh et al. 2003). As endogenous free-fatty acids (FFAs) often
103 activate more than one receptor type (Kostenis 2004) and are metabolised to act as intracellular
104 signalling molecules (Warnotte et al. 1994), they have limited utility as selective tools to establish
105 the pharmacology and functional significance of specific FFA receptors. The recent discovery of
106 selective, small molecule agonists for FFA1 and FFA4 therefore provided a pharmacological
107 advantage over non-selective endogenous FFAs and this study set out to utilise some of these new
108 tool compounds to investigate FFA1 and FFA4 signalling specifically.

109 Apart from FFA1 and FFA4 there are alternative FFA receptors in the gastrointestinal (GI) tract
110 including, FFA2 (formerly GPR43), FFA3 (GPR41) and the acylethanolamide receptor, GPR119,
111 all of which are selectively expressed in L cells together with FFA1 and FFA4 (Overton et al.
112 2006, Hirasawa et al. 2005, Edfalk et al. 2008, Karaki et al. 2006). Both FFA1 (Briscoe et al.
113 2003) and FFA4 (Hirasawa et al. 2008) have the capacity to couple to $G\alpha_{q/11}$, activate
114 phospholipase C (PLC) and elevate Ca^{2+} levels, leading to the release of L cell peptide hormones.
115 Colonic L cells contain peptide YY (PYY), the incretin glucagon-like peptide 1 (GLP-1) plus
116 GLP-2 and oxyntomodulin (OXM) (Habib et al. 2013, Egerod et al. 2012, Cho et al. 2014).
117 Additionally, small intestine L cells express neurotensin and cholecystokinin (CCK) (Egerod et al.
118 2012, Habib et al. 2013). In this study, we utilised the descending colon because this colonic
119 region exhibits a greater degree of PYY-[Y₁](#) signalling compared with the ascending colon (Cox et

120 al. 2001) and L cell density is greater in the distal colon in mouse (Arantes and Nogueira 1997)
121 and human (Adrian et al. 1985). GLP-1 slows gastric emptying and enhances satiety (Little et al.
122 2006) while PYY is a well-known mediator of the ileal and colonic brake (Lin et al. 1996) and a
123 major satiety-inducing agent together with its product, PYY₍₃₋₃₆₎ (Batterham et al. 2002). PYY is
124 also a significant inhibitor of intestinal epithelial ion transport and we have previously utilised this
125 activity to monitor the acute endogenous peptide functionality following L cell-specific
126 stimulation with GPR119 agonists (Cox et al. 2010, Patel et al. 2014) or other L cell mediated
127 mechanisms (Joshi et al. 2013, Panaro et al. 2014). In mouse and human distal colon, local
128 mucosal PYY antisecretory actions are predominantly mediated via epithelial Y₁ receptors with a
129 smaller component of submucosal neuron [Y₂](#) activity (Tough et al. 2006, Tough et al. 2011), that
130 together underpin endogenous PYY paracrine responses. FFA1 and FFA4 are also potential
131 nutrient-sensing receptors in L cells and therefore are possible targets for the treatment of T2DM
132 and obesity (Engelstoft et al. 2008).

133 The FFA1 agonist [TAK-875](#) (Negoro et al. 2010), which in phase II trials lowered blood glucose
134 without apparently increasing GLP-1 levels, was shown to signal via Gα_{q/11} alone, in a manner
135 similar to the proposed endogenous agonist, α-linolenic acid. In type II diabetic rats, TAK-875
136 improved glucose tolerance and enhanced glucose-dependent insulin secretion (Tsujihata et al.
137 2011). Despite the liver toxicity concerns of TAK-875, it significantly improved glycaemic
138 control in diabetic Japanese patients in a phase III study (Kaku et al. 2015). JTT, a novel
139 selective FFA1-targeted agonist (from patent number, WO 2009/054479), TAK-875 and the
140 commercially available FFA1 agonist, [TUG424](#) (Christiansen et al. 2008) were selected to assess
141 the pharmacology of FFA1 in this study.

142
143 FFA4 is also found abundantly in gastric-inhibitory peptide (GIP)-containing K cells of the small
144 intestine, gastric somatostatin (SST)-containing D cells and gastric ghrelin-containing A cells (Ito
145 et al. 2009, Parker et al. 2009, Engelstoft et al. 2013, Egerod et al. 2015). The functional roles of
146 FFA4 include enhanced insulin secretion, GLP-1 secretion (Halder et al. 2013), reduced gastric
147 emptying (Little et al. 2006) and anti-inflammatory effects (Oh et al. 2010). FFA4 knockout (KO)
148 mice exhibited a significantly reduced L cell GLP-1 secretion (Xiong et al. 2013) and K cell GIP
149 secretion (Iwasaki et al. 2015) indicating that signalling via FFA4 in wild type (WT) tissues can
150 cause incretin peptide release. The novel selective agonists selected for this study, AZ423
151 (Compound 34, McCoull et al. 2017) and Metabolex-36 (Ma et al. 2010), show 100-fold
152 selectivity for murine FFA4 compared with FFA1 (Stone et al. 2014). Furthermore, AZ423 and

153 Metabolex-36 have exhibited a reduction in blood glucose excursion in an oral glucose tolerance
154 test (Halder et al. 2013, McCoull et al. 2017). Additionally, the selected commercially available
155 FFA4 agonist, [TUG891](#) is selective for murine and human FFA4 (Hudson et al. 2013). Whether
156 intestinal FFA4 agonism resembles FFA1 activities and is also glucose-sensitive in
157 enteroendocrine L cells has yet to be determined.

158
159 As few functional studies have been performed in native preparations containing L cells, one aim
160 of this study was to determine the potencies and efficacies of the commercially available agonists
161 (TUG424 and TUG891) compared with the non-selective FFA1 and FFA4 agonist [GW9508](#), and
162 importantly the novel selective agonists JTT, TAK-875, AZ423 and Metabolex-36 in mouse
163 colon. We also assessed the selectivity of the FFA1-preferring antagonist [GW1100](#) (Briscoe et al.
164 2006) comparing it with ANT825, a selective FFA1 antagonist (referred to as compound 39 in
165 Waring, 2015) and determined their relative abilities to reveal tonic FFA1 activity. We also
166 determined the involvement of endogenous PYY in these responses in colonic mucosae by
167 blocking peptide activity pharmacologically. Finally, the effects of FFA1 and FFA4 agonism
168 upon colonic motility *in vitro* and *in vivo* were established.

169

170 **Methods**

171 *Mucosal preparation and I_{sc} measurement*

172 Mice (C57BL/6J, 12-20 weeks old, 20-30g, female and male) were procured from Charles River
173 Laboratories (Margate, UK) had free access to standard chow (Rat and Mouse No 3 breeding diet,
174 Special Diets Services, Baintree, UK) and water *ad libitum*. We used mice because the Y₁, Y₂ and
175 Y₄ receptor localisation in the GI tract is the same in this species as in human colon (Cox and
176 Tough. 2001, Tough et al. 2011). Mice were housed in open top conventional cages with Lignocel
177 poplar bedding material along with the appropriate environmental enrichment. A maximum of 5
178 mice were housed in a single cage. Housing rooms were maintained at 20-24 °C, humidity 55% ±
179 10% and 12/12 h light and dark cycle. All animal care and experimental procedures complied with
180 the Animals (Scientific procedures) Act 1986 and were approved by UK Home Office (licence
181 number: PPL70/7887). Mice were killed by cervical dislocation and the whole colon was
182 dissected (noting proximal and distal ends) and placed in fresh Krebs-Henseleit (KH), with the
183 following composition (in mM); NaCl 118, KCl 4.7, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂
184 2.5, and D-glucose 11.1. Mucosae from the descending colon were prepared by removing the

185 overlying smooth muscle layers and associated myenteric plexi by micro-dissection. Mucosal
186 lengths were cut in to 4-6 adjacent pieces, each of which was mounted between two halves of an
187 Ussing chamber (WPI UK, Hitchin, Herts, UK) (exposed area, 0.14 cm²), voltage-clamped at 0
188 mV (DVC1000 and amplifiers; WPI UK, Hitchin, Herts, UK) and equilibrated in oxygenated KH
189 (95% O₂/5% CO₂, at 37°C) for 15 min. Drug additions were made to either the basolateral (bl) or
190 apical (ap) reservoirs and changes in short-circuit current (I_{sc}) were measured continuously.

191

192 *Sidedness, potency and efficacy of the FFA1 and FFA4 compounds*

193 Mucosae were pretreated with vasoactive intestinal polypeptide (VIP, 30 nM, bl) to provide a
194 degree of vectorial epithelial ion secretion upon which subsequent antisecretory signalling is more
195 readily observed (as optimised previously; Tough et al. 2006; Cox et al. 2010). Once VIP
196 responses had reached a maximum, a FFA1 or FFA4 agonist (TUG424 (300 nM), TUG891 (300
197 nM), GW9508 (10 µM)) was added to either reservoir as indicated, to determine the approximate
198 sidedness of the FFA1 and FFA4 responses. Single additions of apical FFA1 or FFA4 agonists
199 were used to construct concentration-response curves and calculate single EC₅₀ values with
200 GraphPad Prism v6.0. Changes in I_{sc} to certain FFA1 agonists (TAK-875, JTT and GW9508) and
201 FFA4 agonists (Metabolex-36, AZ423) were biphasic; the first component (1°) being a transient
202 increase in I_{sc} (within 5 min) followed by a longer-lasting reduction in I_{sc} (the second component,
203 2°) that was extrapolated from the waning VIP signal and reached its maximum within 10-15 min
204 of FFA drug addition. When the biphasic changes in I_{sc} were observed, the 1° and 2° components
205 were analysed separately. To investigate whether there was a neuronal component to FFA1
206 (TAK-875, 200 nM) and FFA4 (Metabolex-36, 100 nM) signalling, mucosae were pretreated with
207 the neurotoxin, tetrodotoxin (TTX, 100 nM, bl) or vehicle (H₂O) for 15 min, followed by VIP and
208 subsequently the FFA1 or FFA4 agonist. The responses to TAK-875 and Metabolex-36 were
209 monitored for 20 min and finally PYY (10 nM, bl) was added as an internal control. Changes in
210 I_{sc} to each drug or peptide were pooled and converted to µA.cm⁻².

211

212 *Establishing the selectivity of FFA1 and FFA4 agonism using antagonists*

213 In order to establish an IC₅₀ of the FFA1 antagonist, ANT825, basal I_{sc} levels were allowed to
214 stabilise before the addition of varying single concentrations (1 nM - 10 µM) of the FFA1
215 antagonist. After 10-15 min, VIP was added, followed by the selected FFA1 agonist, JTT (300

216 nM) and consequent reductions in I_{sc} were recorded and converted to $\mu A.cm^{-2}$. PYY (10 nM) was
217 added finally as an internal control. The antagonist concentration (10 μM , ANT825) that
218 abolished JTT responses was used in subsequent selectivity studies.

219 Blockade of the commercially available FFA1 agonists (TUG424, GW9508 and TAK-875) was
220 confirmed using the antagonists, GW1100 (10 μM) (Briscoe et al. 2006) and ANT825 (10 μM).
221 Here mucosae were pretreated apically for 10 min with the appropriate vehicle (0.1% DMSO) or
222 the chosen FFA1 antagonist prior to VIP addition. TUG424 (100 nM), GW9508 (1 μM) were the
223 FFA1 agonists tested initially, followed by TAK-875 (200 nM) and JTT (300 nM) while TUG891
224 (100 nM), Metabolex-36 (100 nM) and AZ423 (100 nM) were used to preferentially activate
225 FFA4 receptors unless otherwise quoted. Finally, PYY (10 nM) was added as an internal control.
226 ANT825 was chosen as the preferred FFA1 antagonist in further studies.

227

228 *Y₁ and Y₂ receptor antagonist (BIBO3304 and BIIE0246) studies*

229 Tissues were pretreated with previously optimised concentrations of the Y₁ antagonist, BIBO3304
230 (BIBO, 300 nM, bl) or the Y₂ antagonist, BIIE0246 (BIIE, 1 μM , bl) or both, versus their
231 respective DMSO (0.003-0.1 %) controls. After 10-15 min VIP was added and once this response
232 had stabilised, a single apical concentration of the FFA1 or FFA4 agonists was added and the
233 consequent reduction in I_{sc} was converted to $\mu A.cm^{-2}$. PYY and the α_2 -adrenoceptor agonist,
234 UK14,304 (1 μM) were used as internal controls.

235

236 *Mucosal glucose-sensitivity studies*

237 Excised whole colon was placed in fresh glucose-free KH with the following composition (in
238 mM); NaCl 118, KCl 4.7, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, and D-mannitol 11.1.
239 Mucosae were dissected as described above, placed in Ussing chambers and were bathed in KH
240 containing glucose on both sides or replacing glucose with mannitol (at 11.1 mM) on the ap side
241 only. When investigating FFA1 and FFA4 agonism in the presence of lower or higher glucose;
242 the glucose concentration was altered (either 5 mM or 25 mM in KH) on ap and bl sides
243 simultaneously. Mucosal basal I_{sc} levels were allowed to stabilise before the addition of VIP and
244 once stabilised, a single apical addition of the FFA1 or FFA4 agonists was added and consequent
245 reductions in I_{sc} were recorded and converted to $\mu A.cm^{-2}$. Phloridzin (50 μM , ap), the sodium-

glucose co-transporter 1 (SGLT1) inhibitor was used to block apically located SGLT1 and this electrogenic response should be lost when mannitol replaced apical glucose.

248

Faecal pellet propulsion in vitro

The colon (from the caeco-colonic junction to the rectum) was excised, photographed ($t = 0$ min) and bathed in KH at 37°C with either vehicle (0.1% DMSO) or agonist (300 nM TUG424, TUG891, TAK-875 or Metabolex-36) as indicated. After 20 min ($t = 20$ min) each colon length was re-photographed and the distance travelled by the remaining pellets was measured from the rectum, as described previously (Tough et al. 2011). The effects on transit of the agonists were compared in the absence and presence of the FFA1 antagonist, GW1100 (10 μM). In these experiments, the colon was bathed in vehicle (0.1% DMSO) or antagonist, photographed at 20 min, and then bathed in KH containing the agonist of choice for 20 min. The colon was re-photographed at $t = 40$ min. The pellet movement was measured as mean distance travelled relative to the total colonic length and was used to calculate the % colonic transit.

260

Colonic bead excretion in vivo

Mice were acclimatised to handling 3 days prior to experimentation and were fasted 16 h prior to testing, although water was provided *ad libitum*. Plasma glucose before the fast was 10.5 ± 0.9 mmol/l (mean \pm 1 SEM, $n=9$) and 4.8 ± 0.3 mmol/l after fasting ($n=9$). Distal colonic propulsion was measured according to the methods described by Forbes et al (2012). One hour after administration of vehicle, or drug (FFA1 agonist, TAK-875; FFA4 agonist, Metabolex-36, FFA1 antagonist, ANT825) or the positive control (loperamide hydrochloride (HCl) by oral gavage or intraperitoneal (i.p.) injection, mice were placed under 4-5% isoflurane anaesthesia (Isoflurane-VET, Merial Animal Health Ltd, Harlow, UK) and a 2 mm bead was inserted 2 cm intrarectally into the distal colon using blunt tubing (Portex, 1.7×0.4 mm). The mice were subsequently placed into a grid bottom cage, monitored and the time to bead expulsion was measured. Once the bead was excreted, the mouse was killed by cervical dislocation. All drugs were suspended in their respective vehicles and sonicated for 30 min.

274

Upper GI transit in vivo

276 Mice were acclimatized to handling 3 days prior to experimentation and were fasted for 16 h prior
277 to testing. TAK875, Metabolex-36, ANT825, their respective vehicles or loperamide HCl was
278 administered via i.p. injection, 60 min prior to testing. In the experiments that used the SST₂
279 antagonist, CYN 154806, mice were pretreated with the antagonist (3 mg/kg) or saline for 15 min,
280 prior to Metabolex-36 (50 mg/kg, i.p.). A charcoal meal (10% plant charcoal in 5% gum acacia
281 (Tough et al. 2011)) was given by intragastric gavage, and 30 min later the animal was killed by
282 cervical dislocation and the small intestine was isolated from the pyloric to ileocecal junctions.
283 Upper GI transit (UGIT), encompassing gastric emptying and small intestinal motility was
284 determined as previously described (Forbes et al. 2012).

285

286 *Statistical analysis*

287 Pooled responses (as $\mu\text{A}\cdot\text{cm}^{-2}$) are expressed as mean \pm SEM from the numbers of observations as
288 shown, using GraphPad Prism version 6.0 (GraphPad Prism Inc., La Jolla, CA, USA). Single
289 comparisons between groups of data were analysed by unpaired, two-tailed Student's *t*-tests or
290 one-way ANOVA for multiple comparisons with *post hoc* Dunnett's test. *Post hoc* tests were run
291 only when *F* achieved $P < 0.05$ and there was no significant variance inhomogeneity. $P < 0.05$
292 was considered statistically significant. Mice (males and females) were randomised in this study.
293 Additionally, drug treatments added to each mucosal preparation were randomised using the Latin
294 square design technique. Furthermore, vehicle and drug treatments were alternated in the *in vivo*
295 studies for each cohort. Explicit blinding was not performed however; each repeat was compared
296 with one another and the drug-pretreated repeats. Any discrepancies between *n* number cohorts
297 were due to the loss of a single mucosal preparation from a single animal *in vitro*, or a value was
298 removed when it was more than two standard deviations from the mean *in vitro* and *in vivo*. The
299 data and statistical analyses comply with the recommendations on experimental design and
300 analysis in pharmacology (Curtis et al. 2015).

301

302 *Materials*

303 FFA1 (JTT and TAK-875) and FFA4 agonists (AZ423 and Metabolex-36) and the FFA1
304 antagonist (ANT825) were obtained from AstraZeneca, Gothenburg, Sweden. Stock solutions of
305 drugs were dissolved in neat DMSO (at 10^{-2} M or 10^{-3} M). Initial 1:10 dilutions were in neat
306 DMSO and subsequent serial dilutions were in distilled water (excluding the FFA1 agonist, TAK-

875 and the FFA4 agonist, AZ423, where all serial dilutions were performed in distilled water). BIBO3304, (*N*-[(1*R*)-1-[[[4-[(Aminocarbonyl)amino]methyl]phenyl]methyl]amino]carbonyl]-4-[(aminoiminomethyl)amino]butyl]- α -phenyl-benzeneacetamide ditrifluoroacetate), BIIE0246, (*N*-[(1*S*)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b,e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide), GW9508 (4-[(3-Phenoxyphenyl)methyl]amino]benzenepropanoic acid), TUG424, (3-(4-(*o*-Tolylethynyl)phenyl)propanoic acid), TUG891 (4-[(4-Fluoro-4'-methyl[1,1'-biphenyl]-2-yl)methoxy]-benzenepropanoic acid) and CYN 154806 (Ac-(4-NO₂-Phe)-*cyc*(D-Cys-Tyr-D-Trp-Lys-Thr-Cys)-D-Tyr-NH₂) were purchased from Tocris Bioscience (Bristol, UK). GW1100 (4-[5-[(2-ethoxy-5-pyrimidinyl)methyl]-2-[[4-(fluorophenyl)methyl]thio]-4-oxo-1(4*H*)-pyrimidinyl]-benzoic acid,ethyl ester) and loperamide HCl was from Cambridge Bioscience (Cambridge, UK). VIP and PYY were purchased from Cambridge Bioscience (Cambridge, UK) and TTX from Abcam, (Cambridge, UK). VIP, PYY and TTX were dissolved in distilled water. All peptide stocks were stored at -20° C until required and underwent a single freeze-thaw cycle. Phloridzin (1-[2-(β -D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone) was purchased from Sigma-Aldrich (Dorset, UK) and it too was dissolved in distilled water. The following *in vivo* vehicles were all suspended in warm saline, methylcellulose and polyvinylpyrrolidinone (PVP) from Sigma-Aldrich (Dorset, UK), sodium dodecyl sulfate (SDS) from VWR International (Leicestershire, UK), hydroxylpropyl methylcellulose (HPMC) from Alfa Aesar (Lancashire, UK) and Tween-80 from Boston BioProducts (Ashland, USA).

328

329 *Nomenclature of Targets and Ligands*

330 Key protein targets and ligands in this article are hyperlinked to corresponding entries in
331 <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide
332 to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide
333 to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

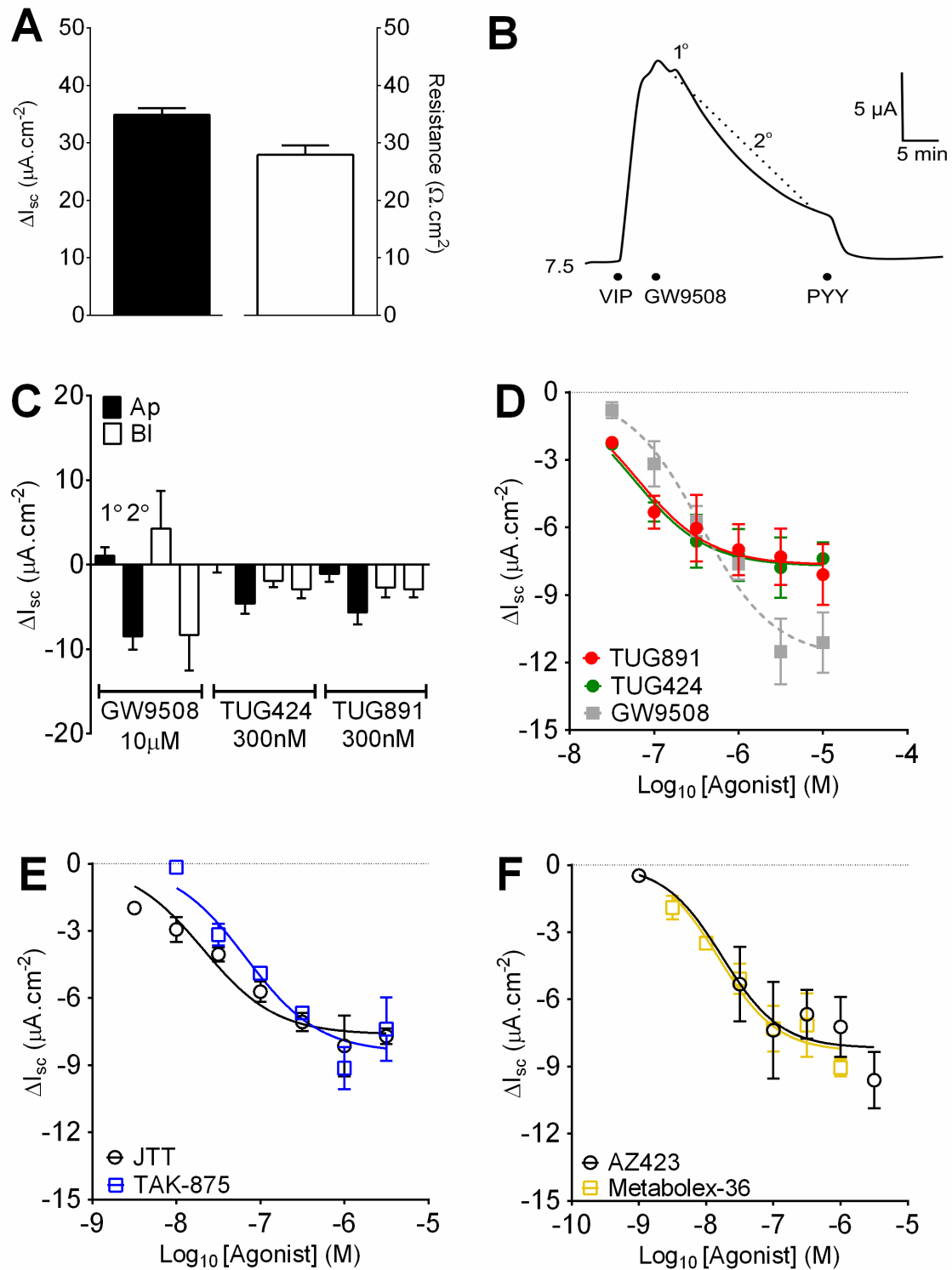
334

335 **Results**

336 *The sidedness, potency and efficacy of FFA1 and FFA4 agonists in descending colon mucosa*

337 First, basal resistance and I_{sc} levels after stabilisation of the colonic mucosae were within ranges
 338 published previously (Figure 1A) (Tough et al. 2011). The activity of the non-selective FFA1 and
 339 FFA4 agonist, GW9508 (Figure 1B), as well as the TUG compounds added apically or
 340 basolaterally induced a biphasic change in I_{sc} . When the reductions in I_{sc} were compared, there
 341 were no significant differences between the response sizes to each agonist added to either side
 342 (Figure 1C). The more selective FFA1 agonists, TAK-875 and JTT and FFA4 agonists,
 343 Metabolex-36 and AZ423 were added apically and induced a biphasic I_{sc} response. The rapid first
 344 I_{sc} component was attributed to the vehicle, 0.1% DMSO ($3.0 \pm 1.1 \mu A.cm^{-2}$, $n=12$) when
 345 compared with the same component for JTT ($1.7 \pm 0.9 \mu A.cm^{-2}$, $n=5$) and Metabolex-36 (3.2 ± 1.7
 346 $\mu A.cm^{-2}$, $n=4$) or 0.01% DMSO ($3.0 \pm 1.1 \mu A.cm^{-2}$, $n=6$) when compared with TAK-875 ($2.5 \pm$
 347 $1.3 \mu A.cm^{-2}$, $n=6$) and AZ423 ($4.6 \pm 1.4 \mu A.cm^{-2}$, $n=6$). Only the later reductions in I_{sc} to varying
 348 concentrations of each FFA1 and FFA4 agonists are shown in Figure 1 D - F. To determine
 349 whether the FFA1 and FFA4 responses were neuronal, we pretreated the mucosae with TTX (100
 350 nM, bl). The responses to TAK-875 ($-11.2 \pm 2.0 \mu A.cm^{-2}$, $n=5$) and Metabolex-36 (-8.3 ± 2.3
 351 $\mu A.cm^{-2}$, $n=5$) were unaffected by TTX compared to their DMSO controls ($-7.5 \pm 1.8 \mu A.cm^{-2}$ and
 352 $-8.1 \pm 2.2 \mu A.cm^{-2}$ respectively, $n=5$), indicating FFA1 and FFA4 responses are most likely
 353 epithelial in origin and their responses are not mediated by TTX-sensitive neurons.

354 The reductions in I_{sc} to each agonist were concentration-dependent, with TUG424 and TUG891
 355 being similarly potent, approximate EC_{50} values were 57.1 nM (24.7 – 131.8 nM) and 62.5 nM
 356 (24.1 – 162.3 nM) respectively (Figure 1D). GW9508 was less potent with an EC_{50} value of 354.8
 357 nM (191.6 – 656.8 nM) and appeared to be slightly more efficacious; however, this was not
 358 significantly different from the TUG agonists (Figure 1D) or the selective FFA1 or FFA4 agonists.
 359 JTT was more potent (EC_{50} of 20.7 nM (12.7 – 34.0 nM)) than TAK-875 (EC_{50} of 67.6 nM (30.6 –
 360 149.4 nM)) and they exhibit similar efficacy (Figure 1E). Furthermore, the FFA4 agonist
 361 Metabolex-36 (EC_{50} of 15.4 nM (7.9 – 30.4 nM)) and AZ423 (EC_{50} of 17.3 nM (3.6 – 83.8 nM))
 362 were similarly potent and efficacious (Figure 1F). In comparison to the selective FFA1 and FFA4
 363 agonists, GW9508 appeared to be a dual agonist.



364

365 **Figure 1. FFA1 and FFA4 responses in descending colon mucosa.** In A: basal I_{sc} and
 366 resistance values after stabilisation of mucosal preparations ($n=20$). In B: representative trace
 367 showing the biphasic nature of apical GW9508 (10 μM) response in descending colon mucosa
 368 after VIP (30 nM) and followed by PYY (10 nM). In C: pooled data showing the primary (1°) and

369 secondary (2°) responses to apical (ap) or basolateral (bl) additions of GW9508 (*n*=5), TUG424
370 (*n*=5) or TUG891 (*n*=5). The 1° responses were recorded within 0-5 min of agonist addition (and
371 were due to vehicle), whereas 2° reductions in *I*_{sc} occurred within 10-15 min. Concentration-
372 response curves for the 2° effects of TUG424 (*n*=5 for all concentrations, except 300 nM (*n*=6))
373 and TUG891 (*n*=5) compared with GW9508 (*n*=5) shown in grey in D; for JTT (*n*=5) and TAK-
374 875 (10 nM (*n*=5), 30 nM (*n*=5), 100 nM (*n*=6), 300 nM (*n*=5), 1 μM (*n*=7) and 3 μM (*n*=8)) in E;
375 and AZ423 (*n*=5) and Metabolex-36 (*n*=5) in F. Bars and points are the means ± 1SEM.

376

377 *GW1100 and ANT825 each inhibit agonist-induced FFA1 responses in the descending colon*

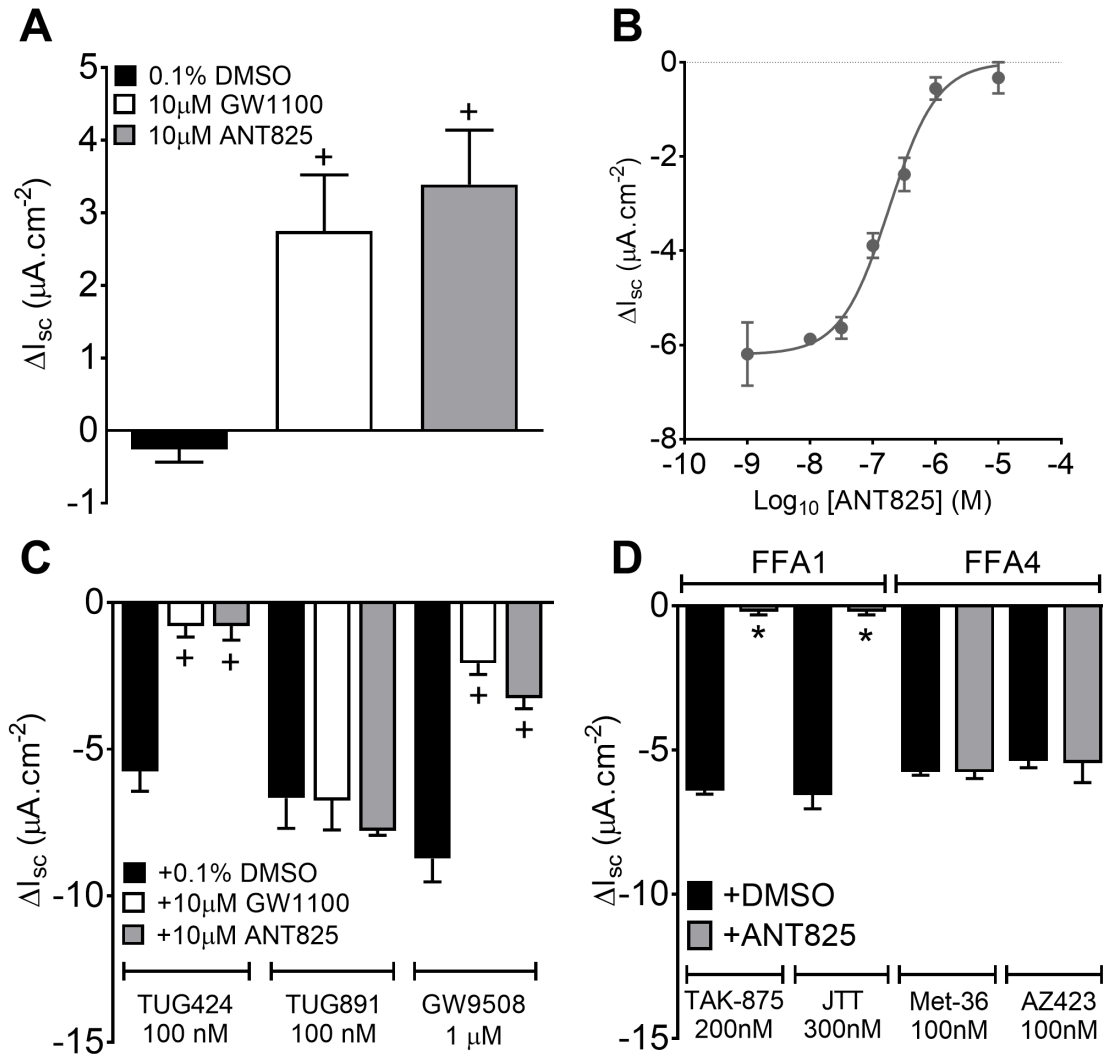
378 The FFA1 antagonists, GW1100 and ANT825 alone revealed a similar degree of FFA1 tonic
379 activity under basal conditions in the distal colon (Figure 2A). ANT825 inhibited JTT responses
380 competitively (Figure 2B), with an IC₅₀ of 219 nM. Optimal blocking concentrations of each
381 FFA1 antagonist, GW1100 or ANT825 revealed the selectivity of TUG424 and GW9508 for the
382 FFA1 receptor. TUG891 responses were not affected by either FFA1 antagonist. Additionally,
383 only part of the GW9508 response was reduced in the presence of ANT825 or GW1100 (Figure
384 2C). The FFA1 selective agonists (TAK-875 and JTT) were significantly inhibited by ANT825
385 whereas the FFA4 selective agonists, Metabolex-36 and AZ423 responses were unaffected (Figure
386 2D).

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393 **Figure 2. Tonic FFA1 activity and inhibition of FFA1 responses by GW1100 or ANT825.** In
394 A: changes in I_{sc} to vehicle, DMSO or the FFA1 antagonists, GW1100 or ANT825 alone. In B:
395 competitive inhibition of the FFA1 agonist, JTT responses by pretreatment with the FFA1
396 antagonist, ANT825. Each point is the mean \pm 1SEM ($n=5$ for all concentrations of JTT except 10
397 μ M ($n=6$)). In C: pooled data shows the selective inhibition of apical TUG424 and GW9508
398 responses, but not TUG891 responses following apical GW1100 or ANT825 treatment compared
399 with respective vehicle controls (+DMSO controls for TUG424 ($n=6$), for TUG891 ($n=5$) and for
400 GW9508 ($n=5$)). In D: selective inhibition of the FFA1 agonists TAK-875 and JTT responses but
401 not the FFA4 agonist, AZ423 and Metabolex-36 (Met-36) responses following apical ANT825
402 treatment (10 μ M, $n=5$). Statistical differences from respective vehicle controls are shown as

403 follows; * $P < 0.05$ (Student's t test) and $^+ P < 0.05$ (one-way ANOVA with Dunnett's *post hoc*
404 test).

405

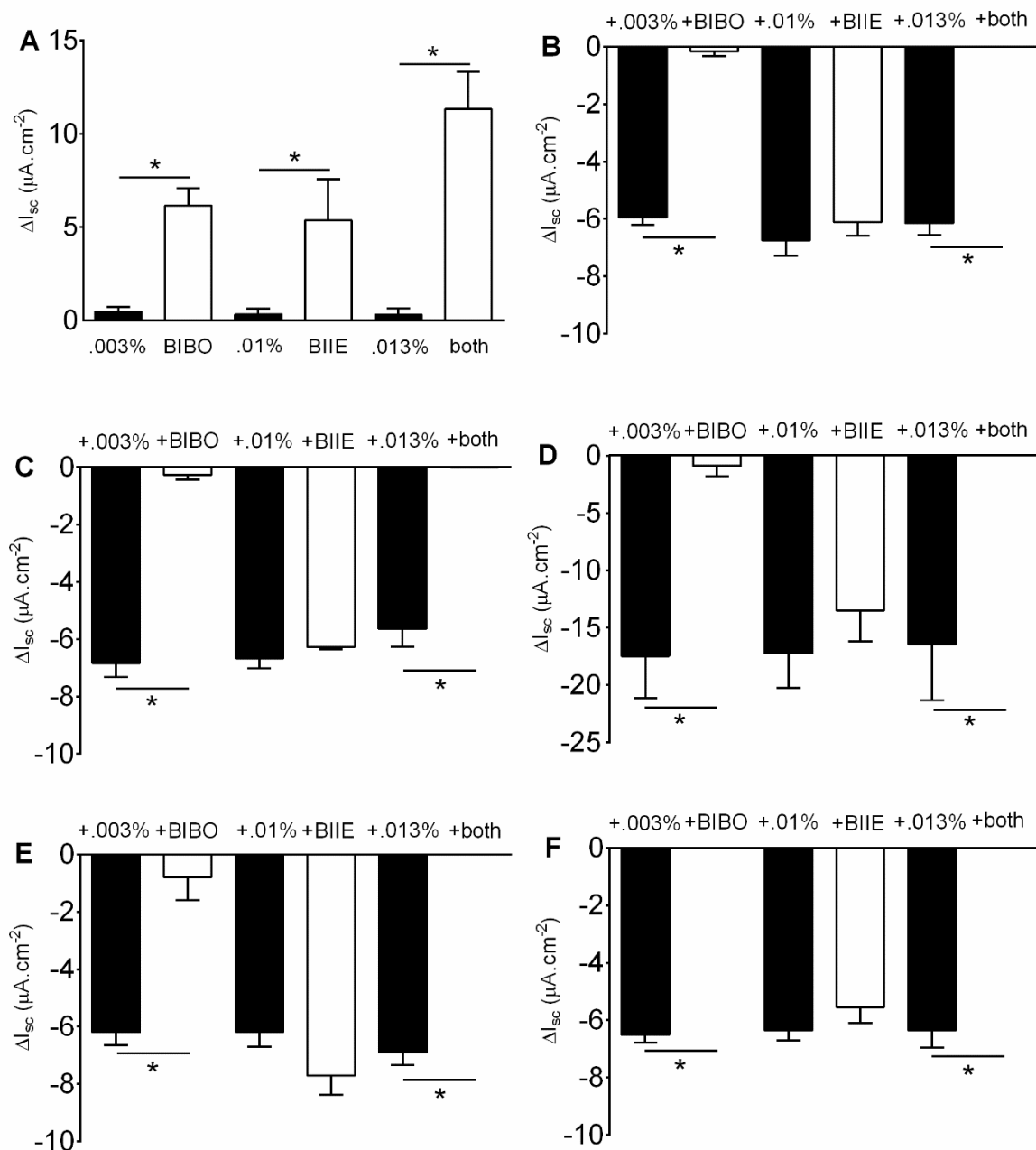
406 *FFA1 and FFA4 colonic responses are BIBO3304 but not BIIE0246 sensitive*

407 Endogenous PYY mediation of the FFA1 (JTT and TAK-875) and FFA4 (AZ423 and
408 Metabolex-36) agonist responses were determined by blocking with the Y_1 antagonist,
409 BIBO3304, the Y_2 antagonist, BIIE0246 or both antagonists together. Each antagonist
410 revealed endogenous PYY- Y_1 and Y_2 tonic activity under basal conditions (Figure 3A)
411 similar to that observed in the mouse colon previously (Tough et al. 2011, Hyland et al.
412 2003). FFA1 responses to JTT (Figure 3B) and TAK-875 (Figure 3C) were abolished by the
413 Y_1 antagonist, indicating PYY- Y_1 signalling predominantly mediates FFA1 responses. The
414 FFA1 responses in the presence of the Y_2 antagonist were slightly reduced, suggestive of a
415 minor role for Y_2 receptors as seen previously (Tough et al. 2011). Notably, there was no
416 influence of the I_{sc} level at the time of adding the FFA1 agonist on subsequent FFA1
417 efficacy. Taken together these observations confirm selective Y_1 -mediation of FFA1
418 responses. Subsequent exogenous PYY responses were also abolished by the Y_1 , but not by
419 the Y_2 antagonist alone, while the combination abolished PYY activity (Figure 3D). The
420 responses to FFA4 agonists, AZ423 (Figure 3E) and Metabolex-36 (Figure 3F) were also
421 abolished by the Y_1 but not Y_2 antagonist and thus both FFA1 and FFA4 responses in the
422 mouse colon are Y_1 receptor mediated.

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426

427 **Figure 3. Y_1 - but not Y_2 -receptor sensitivity of FFA1 and FFA4 agonism in the descending**
 428 **colon.** The effects of the Y_1 (BIBO3304, BIBO, 300 nM) \pm the Y_2 (BIIE0246, BIIE, 1 μ M)
 429 antagonist and corresponding DMSO controls (+.003 %, +.01 %, +.013 %) on baseline I_{sc} levels
 430 are shown in A. The effect of the FFA1 agonists, JTT ($n=5$ for all data groups except +.003% and
 431 +.013% ($n=6$)) and TAK-875 ($n=5$ for all data groups except +BIBO ($n=6$)) in the absence or
 432 presence of Y antagonists are shown in B and C respectively. Exogenous PYY (10 nM) responses
 433 ($n=5$ for all data groups except +.003% and +.013% ($n=6$)) after JTT treatment, in the presence of
 434 DMSO or $Y_1 \pm Y_2$ antagonists are shown in D. The FFA4 agonist's responses, AZ423 ($n=5$) and

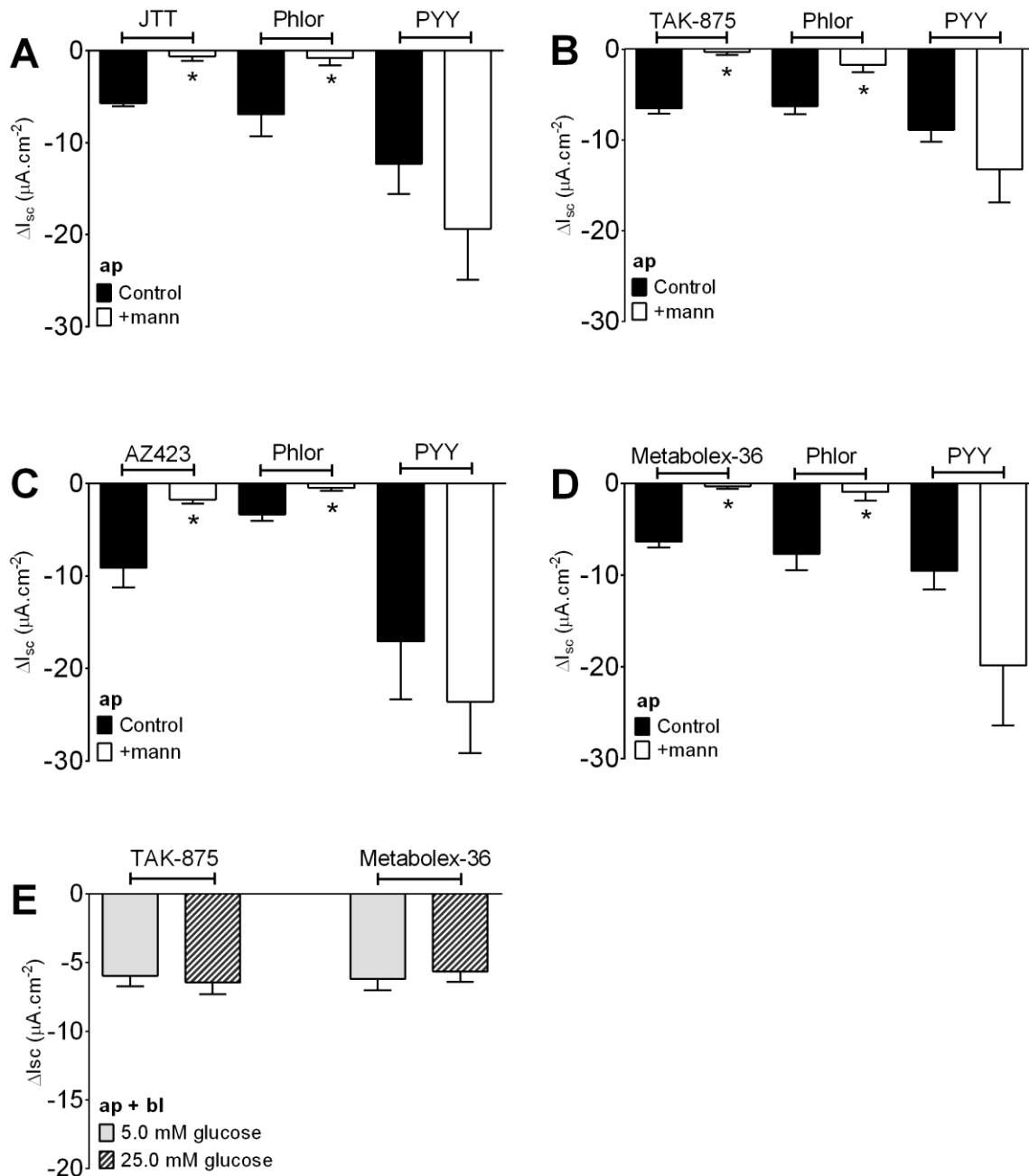
435 Metabolex-36 ($n=5$ for all data groups except $+0.01\%$ ($n=6$)) are shown in E and F respectively.
436 Bars represent the mean \pm 1 SEM. Statistical differences between vehicle controls (0.003 %, 0.01
437 % and 0.013 % DMSO) and respective antagonists BIBO, BIIE or both are shown as follows; * P
438 < 0.05 (Student's t test).

439

440 *FFA1 and FFA4 responses are glucose-sensitive in the descending colon mucosa*

441 In order to establish the glucose-sensitivity of FFA signalling in mucosal preparations, the
442 responses to the FFA1 and FFA4 agonists were compared in the presence or absence of apical
443 glucose. Mannitol replacement of apical glucose abolished the FFA1 responses (Figure 4A & B)
444 and inhibited FFA4 activity (Figure 4C & D) compared to vehicle. Internal controls using
445 phloridzin (Phlor, added ap) decreased the I_{sc} , but as expected only in the presence of glucose
446 (because SGLT1 requires glucose to function and is targeted apically). Thus, FFA1 and FFA4
447 receptors can be activated in a glucose-sensitive manner. In contrast, PYY responses were not
448 glucose-sensitive (Figure 4A-D). Furthermore, TAK-875 and Metabolex-36 responses were not
449 affected when the glucose concentration (ap + bl) was varied to represent a hypoglycaemic (5.0
450 mM) or hyperglycaemic (25.0 mM) environment (Figure 4E).

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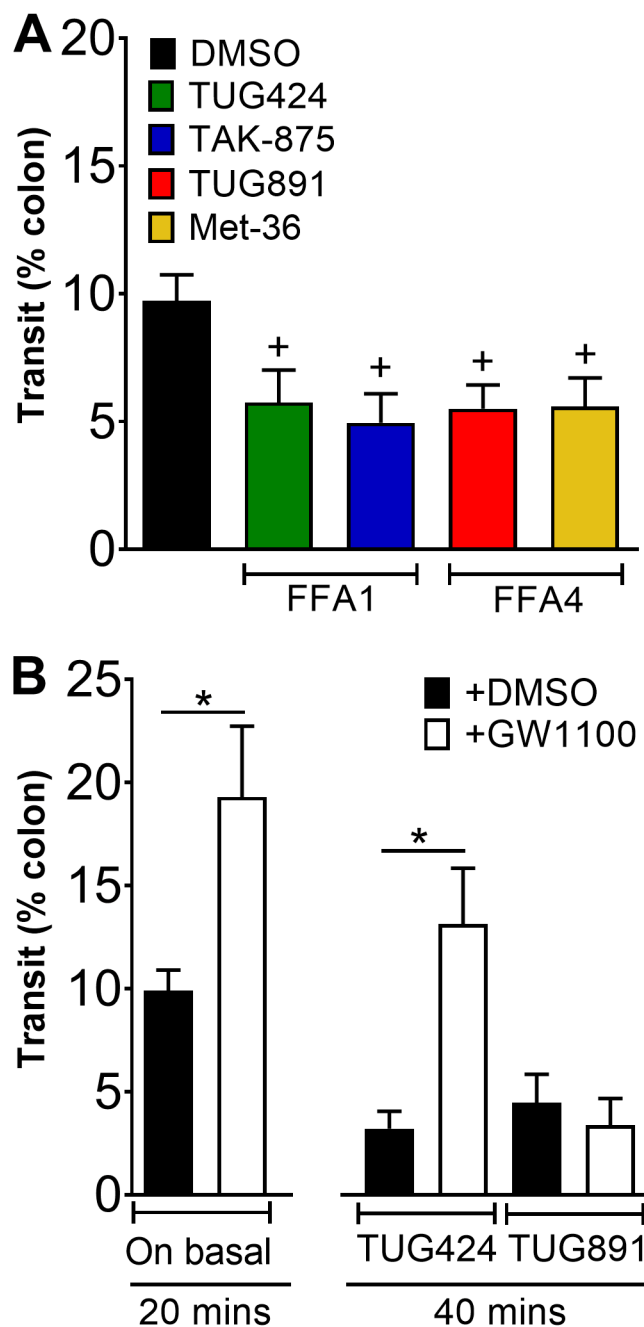
453 **Figure 4. Glucose-sensitivity of FFA1 and FFA4 agonism in the descending colon.** Glucose-
 454 sensitivity of apical FFA1 agonists, JTT (300 nM, $n=5$) in A, TAK-875 (200 nM, $n=5$) in B and
 455 apical FFA4 agonists, AZ423 (100 nM, $n=5$) in C and Metabolex-36 (100 nM, $n=5$) in D, in the
 456 presence (black bars) and absence (white bars) of 11.1 mM glucose. Control mucosae were
 457 bathed in glucose both sides whereas, mannitol (+ mann, 11.1 mM) replaced glucose apically
 458 only. Phloridzin (Phlor, 50μM, apically only) and PYY (10 nM) responses are also shown. In E:
 459 TAK-875 (200 nM, $n=5$) and Metabolex-36 (100 nM, $n=5$) antisecretory responses in the presence
 460 of apical and basolateral (ap + bl) 5.0 mM glucose or 25.0 mM glucose. Bars represent the mean -

1SEM. Statistical differences between agonist or phloridzin responses in the presence or absence of glucose, are shown as follows; * $P < 0.05$ (Student's t test).

Caeco-colonic transit is inhibited by FFA1 and FFA4 agonists

Since FFA1 and FFA4 mucosal responses in the colon were mediated by PYY and this endogenous peptide is known to slow colonic transit (Tough et al. 2011), we next assessed whether the FFA1 and FFA4 agonists reduced transit at single optimal concentrations (300 nM; Figure 5A). In isolated colons, TUG424, TUG891, TAK-875 and Metabolex-36 significantly decreased transit in comparison to vehicle-treated (DMSO, 0.1%) controls.

Since GW1100 (10 μ M) inhibited FFA1 responses in colonic mucosa, we set out to determine whether GW1100 alone could increase basal faecal transit and reverse the effect of the FFA1 agonist on colonic transit, *in vitro*. Figure 5B shows that GW1100 alone significantly increased basal colonic transit, again indicating endogenous FFA1 inhibitory tone. After 20 min pretreatment with the FFA1 antagonist, GW1100 the effect of TUG424 and TUG891 was assessed in the presence and absence of GW1100. In the presence of GW1100, the inhibitory effect on colonic transit of TUG424 was reversed, but GW1100 had no effect on TUG891 (FFA4) activity, showing FFA1 selectivity (Figure 5B).



506 **Figure 5. Colonic transit is slowed by FFA1 and FFA4 agonists in isolated colons *in vitro*.**
 507 Colonic transit is slowed by 300 nM of TUG424 ($n=6$), TAK-875 ($n=6$), TUG891 ($n=6$) and
 508 Metabolex-36 (Met-36, $n=6$) in A at $t=20$ min. In B: the effects of FFA1 antagonist GW1100 (10
 509 μ M) are compared on basal faecal transit at $t=20$ min and on subsequent inhibition of transit
 510 following 20 min treatment with TUG424 or TUG891 at $t=40$ min ($n=5$). Each bar represents
 511 the mean \pm 1SEM. Statistically significant differences from controls were; * $P < 0.05$ (Student's t
 512 test) and $^+ P < 0.05$ (one-way ANOVA with Dunnett's *post hoc* test).

513

514 *Colonic bead propulsion is slowed by Metabolex-36 not TAK875 or ANT825 in vivo*

515 As the FFA1 and FFA4 agonists, (TAK-875 and Metabolex-36 respectively) slowed transit in
516 isolated colons *in vitro*; we assessed the ability of these agonists and loperamide HCl to slow
517 colonic transit *in vivo*, comparing oral administration versus i.p. injection. Additionally, we
518 investigated whether ANT825 had the potential to reveal FFA1 tone *in vivo*. Oral administration
519 of the positive control, loperamide HCl significantly slowed transit compared to its vehicle
520 control. Two doses of Metabolex-36 were assessed orally; the lower dose had no effect on transit
521 whereas the higher dose significantly slowed transit. Furthermore, oral gavage of TAK-875 and
522 ANT825 had no effect on colonic transit (Figure 6A). Both loperamide HCl and Metabolex-36
523 significantly slowed colonic transit when given via i.p. injection whereas TAK-875 and ANT825
524 had no effect (Figure 6B). As i.p. injection of loperamide HCl slowed transit more efficiently in
525 comparison to oral gavage, we used this route of administration for each drug in the subsequent *in*
526 *vivo* studies.

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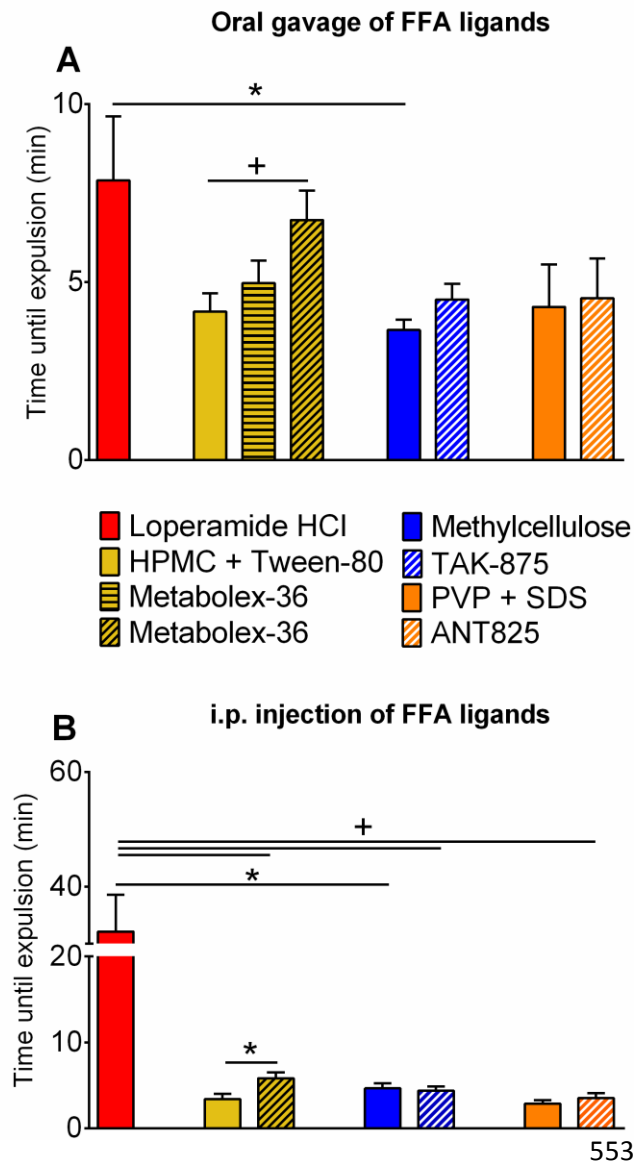
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554 **Figure 6. The effect of oral gavage versus i.p. injection of FFA ligands and loperamide HCl**
555 **on colonic bead expulsion *in vivo*.** The effect on colonic bead expulsion (expressed as time until
556 bead expelled (min)) after oral gavage or i.p. injection of loperamide HCl (10 mg/kg, red bar,
557 $n=11$) compared with its vehicle control, 0.5% methylcellulose (blue bar, $n=11$); the FFA4
558 agonist, Metabolex-36 at two doses: 6 mg/kg (yellow bar with black horizontal stripes, $n=6$) and
559 50 mg/kg (yellow bar with black diagonal stripes, $n=10$) compared with their vehicle control,
560 0.5% hydroxylpropyl methylcellulose (HPMC) + 0.1% Tween-80 (yellow bar, $n=10$); the FFA1
561 agonist, TAK-875 (27 mg/kg, blue bar with white diagonal stripes, $n=11$) compared with its
562 vehicle control (0.5% methylcellulose, blue bar) and finally the FFA1 antagonist, ANT825 (29
563 mg/kg, orange bar with white diagonal stripes, $n=7$) compared with its vehicle (0.25%
564 polyvinylpyrrolidone (PVP) + 0.05% sodium dodecyl sulfate (SDS), orange bar, $n=7$). Each bar

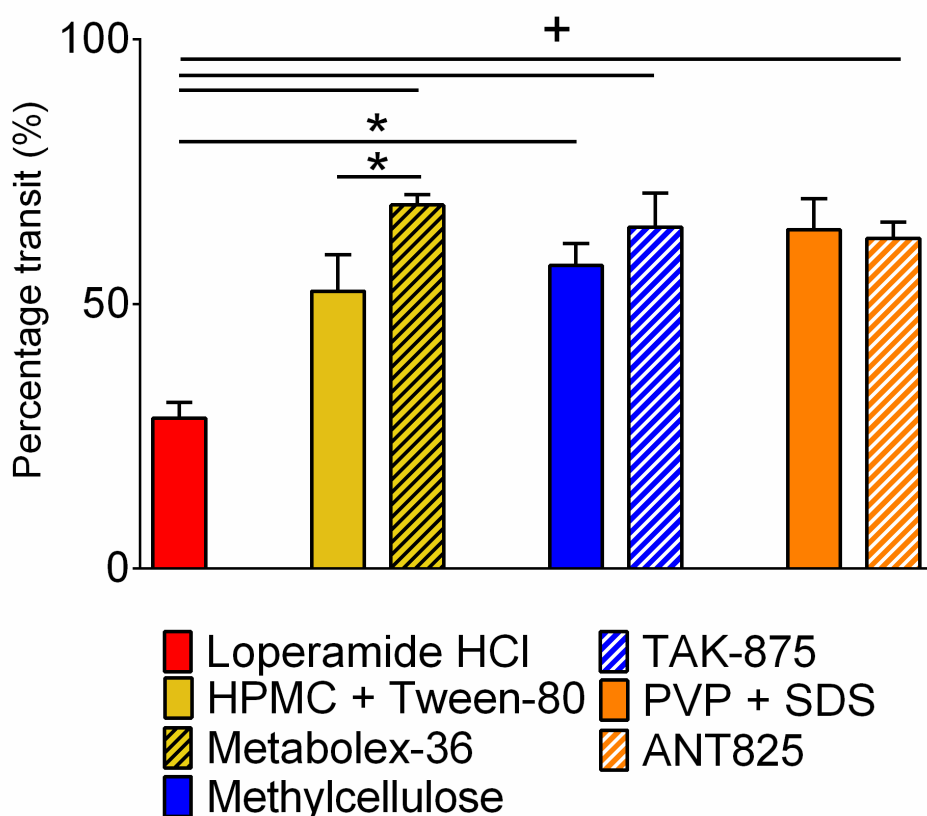
565 represents the mean + 1SEM. Statistically differences from controls were; * $P < 0.05$ (Student's t -
566 test). Differences between the oral doses of Metabolex-36 from its control and additionally
567 between loperamide HCl (i.p.) and the FFA drugs were; + $P < 0.05$ (one-way ANOVA with
568 Dunnett's *post hoc* test).

569

570 *Upper GI transit is increased by Metabolex-36 in vivo*

571 Here we investigated whether the FFA agonists (i.p.) and loperamide HCl could slow intestinal
572 transit and additionally whether ANT825 could increase intestinal transit (revealing FFA1 tone),
573 after mice were given a charcoal meal (as described in the methods). The positive control,
574 loperamide HCl significantly slowed GI transit, as expected compared to its vehicle control.
575 Unexpectedly, Metabolex-36 significantly increased intestinal transit whereas TAK-875 and
576 ANT825 had no effect (Figure 7).

577



589

Figure 7. The effect of an i.p. injection of loperamide HCl and FFA ligands on UGIT *in vivo*.

The intestinal transit of a charcoal meal (expressed as a percentage of the small intestine length) after i.p. injection of the positive control, loperamide HCl (10 mg/kg, red bar, $n=6$) compared with its vehicle control, 0.5% methylcellulose (blue bar, $n=6$); the FFA4 agonist, Metabolex-36 (50 mg/kg, yellow bar with black diagonal stripes, $n=6$) compared with its vehicle control, 0.5% hydroxypropyl methylcellulose (HPMC) + 0.1% Tween-80 (yellow bar, $n=6$); the FFA1 agonist, TAK875 (27 mg/kg, blue bar with white diagonal stripes, $n=5$) compared with its vehicle control (0.5% methylcellulose, blue bar) and the FFA1 antagonist, ANT825 (29 mg/kg, orange bar with white diagonal stripes, $n=5$) compared with its vehicle (0.25% polyvinylpyrrolidone (PVP) + 0.05% sodium dodecyl sulfate (SDS), orange bar, $n=5$). Each bar represents the mean + 1SEM. Statistically differences from controls were; * $P < 0.05$ (Student *t*-test) whereas differences between loperamide HCl and FFA drugs were; + $P < 0.05$ (one-way ANOVA with Dunnett's *post hoc* test).

SST₂ inhibition has no effect on Metabolex-36 induced UGIT effect *in vivo*

As Metabolex-36 increased UGIT *in vivo*, we investigated whether this was due to an inhibitory FFA4 effect (via a $G\alpha_i$ -mediated mechanism) on somatostatin-containing D cells in the GI tract. This inhibition would result in disinhibition and ultimately a pro-motile effect, as somatostatin is a known inhibitor of GI motility. Mice were pretreated with the SST_2 antagonist, CYN 154806 or saline before the effect of Metabolex-36 was tested. CYN 154806 + Metabolex-36 had no effect on the UGIT of the charcoal meal, in comparison to mice pretreated with saline (Figure 8).

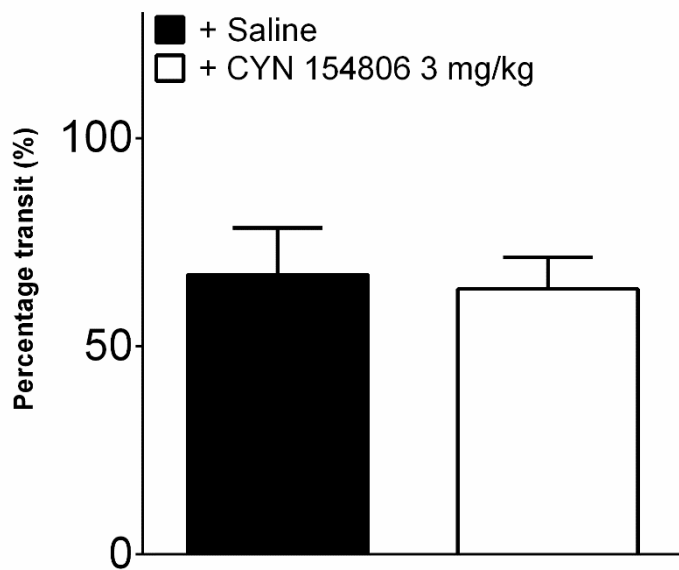


Figure 8. CYN 154806 had no effect on the Metabolex-36 induced increase in UGIT *in vivo*.

The effect of Metabolex-36 (50 mg/kg, i.p. injection) on UGIT in the absence (saline, $n=5$) or presence ($n=5$) of the SST₂ antagonist, CYN 154806. Bars represent mean + 1 SEM.

Discussion

The improved selectivity of FFA1 and FFA4 agonists versus commercially available agonists

FFA1 and FFA4 agonism was independent of the surface of administration and the time-dependence of I_{sc} changes was similar, suggesting that these receptors are located on both the apical and basolateral surfaces. However, it should be noted that all the drugs used were lipid soluble requiring DMSO as a vehicle therefore, drugs added on the apical surface could activate FFA receptors on the basolateral side and vice versa. Interestingly, Christensen et al. (2015) showed preferential but not exclusive vascular (basolateral) FFA1-induced GLP-1 secretion using endogenous and synthetic agonists, in rat small intestine. We utilised apical administration and the reductions in I_{sc} we observed occurred within the same time frame as other apically located L cell $G\alpha_q$ -coupled receptors, some activated by water-soluble compounds (Joshi et al. 2013, Forbes et al. 2015, Alamshah et al. 2016) thus indicating apical receptors.

GW9508 is reported to act via FFA1 and FFA4 with 100x higher affinity for FFA1 than FFA4 (Briscoe et al. 2006). GW9508 is a dual agonist at the FFA1 and FFA4 and this may explain its

645 slightly greater efficacy than more selective agonists we used. We found that the selective FFA1
646 and FFA4 agonists exhibited a similar potency which was more potent than the potency of
647 GW9508. Both FFA4 agonists, Metabolex-36 and AZ423 have >100-fold higher selectivity for
648 FFA4 in comparison to the FFA1. This phenomenon of higher potency but lower efficacy has
649 been observed previously with a GPR119 agonist, PSN-GPR119 (Patel et al. 2014) and may
650 indicate acute receptor desensitisation.

651 FFA1 agonists are preferentially $G\alpha_{q/11}$ -linked and recent evidence suggests that ' $G\alpha_{q/11}$ -only'
652 FFA1 agonists (e.g. TAK-875) stimulate the release of incretin hormones, GLP-1 and GIP, with
653 reduced efficacy compared to FFA1 agonists that signal via $G\alpha_{q/11}$ and $G\alpha_s$ pathways; to cause a
654 more robust release of GLP-1 and GIP (Hauge et al. 2015). Our FFA1 agonists appear to be
655 $G\alpha_{q/11}$ -coupled preferentially as mucosal responses were transient in comparison with $G\alpha_s$ -coupled
656 L cell signalling e.g. GPR119 or MC4 (Cox et al. 2010, Panaro et al. 2014).

657

658 *FFA1 antagonism reveals FFA1 mucosal and anti-motility tone and agonist-specificity.*

659 The FFA1 antagonists, ANT825 and GW1100 revealed, for the first time, a degree of endogenous
660 FFA1 tonic activity in colonic mucosa. The degree of tonic activity was similar to that observed
661 with the MC4 receptor antagonist, HS014 ($3.6 \mu A.cm^{-2}$) (Panaro et al. 2014). Since endogenous
662 FFA1 and MC4 agonism stimulates PYY release from L cells, blockade of these receptors inhibits
663 endogenous PYY release. Crivellato et al (2002) demonstrated that enteroendocrine cells in
664 mouse colon undergo piecemeal degranulation, hormone release that may be related to the tonic
665 activity we observe in mucosa. Endogenous lipids, natural ligands of the FFA1 (Itoh et al. 2003)
666 may be responsible for the observed tonic activity via FFA1.

667 The potency of the FFA1 competitive antagonist, ANT825 was not dissimilar from its potency
668 (pEC_{50} of 6.8) obtained in a human embryonic kidney 293 cell line expressing human FFA1,
669 measuring inositol monophosphate (Waring 2015). We found GW1100 (pIC_{50} of 5.99; (Briscoe et
670 al. 2006)) abolished TUG424 responses but not TUG891 responses, showing FFA1 selectivity as
671 seen previously (Briscoe et al. 2006). Like GW1100, ANT825 abolished FFA1 responses but
672 both FFA1 antagonists' also partially inhibited GW9508 responses, indicating that at this
673 concentration ($1 \mu M$); GW9508 exerts a dual agonism via FFA1 and most likely FFA4. Briscoe et
674 al (2006) revealed that GW1100 had no effect on the ability of GW9508 to activate FFA4. We
675 conclude that GW9508 is a dual FFA1 and FFA4 agonist.

676 FFA1 (TUG424, TAK-875) and FFA4 (TUG891, Metabolex-36) agonists attenuated colonic
677 motility *in vitro* to a similar degree as seen previously with MC4 receptor activation and GPR119
678 agonism (Panaro et al. 2014). They decreased colonic motility presumably via the release of
679 PYY, which mediates the colonic brake. Pretreatment with the FFA1 antagonist, GW1100
680 revealed basal colonic mucosal tone (as seen with Ussing chamber studies) indicative of
681 endogenous FFA1 activity. In the presence of the FFA1 antagonist GW1100, the colonic brake
682 induced by the FFA1 agonist (TUG424) was disinhibited and therefore colonic transit increased
683 whereas transit induced by FFA4 agonist (TUG891) remained unaffected, showing selectivity of
684 the FFA1 agonist.

685 As the FFA1 agonist, TAK875 and the FFA4 agonist, Metabolex-36 slowed transit *in vitro*; we
686 investigated their ability to slow transit *in vivo*. Following the overnight fast, plasma glucose
687 levels were reduced to 5 mM. However, we established that FFA1 and FFA4 signalling is
688 unchanged at 5 mM compared with 11.1 mM glucose as seen previously for GPR119 agonism
689 (Patel et al. 2014). Metabolex-36 slowed colonic transit significantly presumably via PYY,
690 whereas TAK-875 failed to show significant inhibition of bead colonic transit when administered
691 orally or via i.p. injection. This apparent lack of efficacy may be due to TAK-875's partial
692 agonism or its administration as a suspension. In our *in vitro* transit studies, the isolated colon is
693 severed from the central nervous system (CNS) and TAK-875 acts directly upon colonic FFA1
694 receptors. In the *in vivo* model, it's possible that the ability of TAK-875 to release PYY is not
695 sufficient to alter colonic motility, with the added complication of modulatory CNS pathways. In
696 addition, there are concerns in the literature that isoflurane interferes with GI transit. In 2005,
697 Torjman and colleagues reported that 6 min exposure to isoflurane in rats slows UGIT by 50%
698 (Torjman et al. 2005). In our study, mice received a 2 min exposure of isoflurane 1 h after the
699 drug, vehicle or positive control were administered, followed by bead insertion and excretion.
700 Therefore, we conclude that this brief exposure of isoflurane in our protocol (at the time of
701 maximal drug exposure) should have minimal effect on colonic motility. Furthermore, we note
702 that there are no differences in colonic transit rates between different vehicle controls (oral gavage
703 vs i.p.) in this study, compared with those in a previous investigation (Forbes et al. 2012).

704 Unexpectedly, Metabolex-36 was pro-motile in the upper intestine. FFA4 is expressed on I cells,
705 K cells and L cells causing release of CCK, GIP and PYY (and GLP-1) respectively, via $G\alpha_q$ -
706 signalling (Iwasaki et al. 2015, Hirasawa et al. 2005, Tanaka et al. 2008). These peptides inhibit
707 gastric emptying and small intestinal transit and additionally, PYY is responsible for both ileal and
708 colonic brakes (Spiller et al. 1984, Spiller et al. 1988). However, recently FFA4 has been

709 identified in gastric ghrelin and somatostatin cells, signalling via $G\alpha_i$ proteins to inhibit peptide
710 release (Engelstoft et al. 2013, Egerod et al. 2015). Somatostatin is known to inhibit motility and
711 increase the interval between migrating myoelectric complexes (MMC), therefore slowing transit.
712 We hypothesised that Metabolex-36 increased intestinal motility by inhibiting somatostatin release
713 and tested this possibility with the SST₂ antagonist, CYN 154806 at an effective dose used
714 previously in rat small intestine, *in vivo* (Booth et al. 2001). However, CYN 154806 was
715 ineffective (at 3 mg/kg) and we conclude that either the antagonist dose used was too low in the
716 mouse, or Metabolex-36 produces a pro-motile effect via an alternative FFA4 mechanism.

717

718 *PYY-Y₁ but not PYY-Y₂ mediation of FFA1 and FFA4 signalling in mouse colonic mucosa*

719 FFA1 and FFA4 agonist responses in the mouse colon were Y₁ receptor mediated. Additionally,
720 these responses were unaffected by TTX indicating a predominantly epithelial origin for FFA1
721 and FFA4 activity, not involving TTX-sensitive submucosal neurons. The degree of antisecretory
722 tone mediated by the Y₁ and Y₂ receptors was similar to that shown previously in mouse colon
723 (Hyland et al. 2003, Tough et al. 2011). The FFA1 and FFA4 signalling in colonic mucosa are
724 mediated by endogenous PYY acting on local epithelial Y₁ receptors. The epithelial Y₁ mediation
725 and lack of any Y₂ (neuronal) involvement is consistent with our previous findings for other L
726 cell-enriched receptors e.g. MC4 (Panaro et al. 2014), GPR119 (Patel et al. 2014), FFA2 (Forbes
727 et al. 2015) and the calcium-sensing receptor (Joshi et al. 2013). There appears to be no GLP-1
728 mediation of FFA1 and FFA4 responses in this study. GLP-1 has a half-life of 1.5 min which
729 limits the ability to detect its response, if any. Additionally, we have discovered that the GLP-1
730 response can vary across the colon, with largest GLP-1 responses observed in the ascending colon
731 and smallest in the descending colon (Tough et al. 2017). Here we have used descending colon
732 and in combination with the short half-life of GLP-1, this may explain why we do not see a GLP-1
733 mediated response.

734

735 *FFA1 and FFA4 responses in mouse colon mucosa are glucose-sensitive*

736 Our studies demonstrate for the first time that activation of FFA1 and FFA4 responses in native
737 tissues are glucose-sensitive as seen in previous investigations of L cell-specific activation by
738 GPR119 (Cox et al. 2010), MC4 receptor (Panaro et al. 2014), the activation of the calcium-
739 sensing receptor by L-glutamine (Joshi et al. 2013) and FFA2 agonism (Forbes et al. 2015).

740 Theoretically these agonists should not therefore cause hypoglycaemia *in vivo*, a disadvantage of
741 certain anti-diabetic drugs currently on the market. FFA1 and FFA4 receptors are currently
742 clinically favourable targets as they are not only co-expressed in GI L cells but differentially
743 expressed in the pancreas. FFA1 is expressed in pancreatic β -cells (Itoh et al. 2003) and FFA4 has
744 recently been discovered in somatostatin-containing delta cells (Stone et al. 2014). PYY and
745 GLP-1 released from the L cells should enhance satiety (an advantage for patients with associated
746 obesity) and elevate insulin release respectively via activation of GLP-1 receptors on pancreatic β
747 cells. Furthermore, an FFA1 agonist could simultaneously directly elevate insulin release after a
748 meal. This combination of indirect and direct FFA1 effects should improve glucose tolerance
749 without hypoglycaemia.

750

751 *Final conclusions*

752 We have shown that selective FFA1 and FFA4 agonism is PYY- Y_1 receptor mediated and
753 glucose-sensitive in mouse descending colon mucosa. These compounds would limit potential
754 hypoglycaemia, a side effect of certain commercially available anti-diabetic drugs. ANT825 and
755 GW1100 revealed tonic FFA1 activity and the possibility of FFA1 endogenous ligands causing
756 PYY release. Furthermore, we have shown for the first time that agonism of FFA1 and FFA4
757 inhibits colonic transit *in vitro* and FFA4 agonism induces regional differences in transit *in vivo*.
758 In colonic L cells agonism of FFA4 is presumably G_{α_q} -mediated and leads to antisecretory
759 mucosal responses that slows colonic transit, but leads to upper GI pro-motile activity *in vivo* in
760 the mouse, which remains unresolved. FFA1 and FFA4 co-agonism may be beneficial
761 therapeutically and would more closely mimic the combinatorial activity of long chain fatty acids
762 in the diet.

763

764 *Author contributions*

765 RM and IRT performed and analysed the experiments and contributed with HMC to the design
766 and interpretation of the data, and writing of the manuscript. DMS and MS provided AstraZeneca
767 in-house data and contributed to the design and interpretation of the work. MS and HMC had the
768 project idea and got funding. RM, IRT, DMS, MS and HMC reviewed and approved the final
769 manuscript version and agreed to be accountable for all aspects of the work.

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